REMARKS

Upon entry of the foregoing amendments, Claims 1, 2, 6, 7, 9 and 11-17, and 20-28 will be pending. Applicants have cancelled Claims 18 and 19 without disclaimer of, or prejudice to, the subject matter contained therein. Applicants maintain that the cancellation of a claim makes no admission as to its patentability, and reserve the right to prosecute the subject matter of the cancelled claims in this or any other patent application. Applicants have amended Claims 2 and 20, and have added new Claims 26-28. The amendments add no new matter and are fully supported by the specification and claims as originally filed.

Claims 1, 2, 6, 7, 9, 11, 13-15 and 18-25 were examined in the Office Action mailed February 19, 2010. Applicants respond below to the specific rejections set forth in the Office Action. For the reasons below, Applicants respectfully traverse the rejections.

Rejection Under 35 U.S.C. § 112, first paragraph – Written Description (Claims 18 & 19)

The Examiner has rejected Claims 18 and 19 as allegedly not being adequately described in the specification. Specifically, the Examiner asserts that the phrase "a solution prepared from an evaporated mixture of DDA, DODA, or DC Chol and an apolar fraction of a total lipid extract of BCG, M. microti, M. tuberculosis, M. vaccae, M. bovis or M. africanum and a solvent," is not adequately described. (Office Action, at 4).

Without acquiescing to the Examiner's position, and solely in the interest of advancing the prosecution of the instant case, Applicants have cancelled Claims 18 and 19. The amendment renders the Examiner's rejection moot, and Applicants respectfully request withdrawal of the rejection.

Rejection Under 35 U.S.C. § 112, first paragraph – Written Description (Claims 20-23)

The Examiner has rejected Claims 20-23 as allegedly not being adequately described in the specification. Specifically, the Examiner asserts that the phrase "a solution prepared from an evaporated mixture of DDA, DODA, or DC Chol and an apolar fraction of a total lipid extract of BCG, M. microti, M. tuberculosis, M. vaccae, M. bovis or M. africanum and a solvent," is not adequately described. (Office Action, at 5).

As amended, Claim 20, and by extension dependent Claims 21-23, recite "[a]n adjuvant consisting essentially of a mixture of a solvent, DDA, and an apolar fraction of a total lipid extract of BCG, *M. microti*, *M. tuberculosis*, *M. vaccae*, *M. bovis* or *M. africanum*." Applicants respectfully submit that the presently claimed adjuvant is specifically described in the specification. In particular, paragraph [0013] of the specification describes extracting apolar lipids using a solvent, bringing the lipid extract into aqueous suspension, and mixing the solution with DDA. As such, it is clear from at least paragraph [0013] that Applicants were in possession of the presently claimed embodiment.

In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

Claim Objection

The Examiner has objected to Claim 2, for containing a typographical error. Applicants' amendment to Claim 2 addresses and overcomes the objection. Applicants respectfully request withdrawal of the objection accordingly.

Rejection Under 35 U.S.C. § 103(a) – Claims 1, 2, 6, 7, 11, 13-15, and 24-25

The Examiner has rejected Claims 1, 2, 6, 7, 11, 13-15 and 24-25 as allegedly being unpatentably obvious over U.S. Patent Application Publication No. 2002/0044951 to Liu et al. ("Liu"), in further view of Dascher et al. (2003) *Int. Immunol.* 15(8):915-925 ("Dascher"), U.S. Patent No. 6,218,166 to Ravindranath et al. ("Ravindranath") and Lindabald et al. (1997) *Infect. Immun.* 65(2):623-629 ("Lindabald"). According to the Examiner, Liu discloses an adjuvant comprising surfactants and an apolar fraction or part of total mycobacterial lipid extract, *e.g.* from *M. tuberculosis*. The Examiner concedes that Liu does not disclose an adjuvant comprising DDA, the particular mycobacterium apolar lipids recited in Claim 2, or compositions that further comprise an antigenic component comprising an antigenic epitope, as recited in Claim 9. The Examiner asserts that Dascher teaches a *Mycobacterium* vaccine that includes DDA and cholesterol as carrier lipids. The Examiner asserts that Ravindranath teaches adjuvant-incorporated cell compositions having an immunopotentiating agent inserted into the cellular membrane or intracellular compartment of the cells. According to the Examiner, it would have

been *prima facie* obvious at the time the invention was made to "modify the adjuvant (disclosed by Liu et al) and to incorporate DDA (disclosed by Dascher et al)," (Office Action, at 8), since Lindabald allegedly teaches that DDA increases that efficiency of immune responses to subunit vaccines. The Examiner argues that "one would have a reasonable expectation of success because "an adjuvant comprising a surfactant and an apolar fraction (as disclosed by Liu et al.) is well known in the art." (Id.). Next, the Examiner asserts that Ravindranath teaches useful adjuvants comprising whole or part of cell phenolic glycolipids that can be conjugated to cellular vaccines. The Examiner concludes by stating that since the use of phenolic glycolipids as adjuvants in vaccine compositions is known and "leads to predictable results," it would have been obvious to use the same in the "adjuvants" of Liu, even absent a express statement of motivation. (Id.). The Examiner concludes that as such, the references render the claims *prima facie* obvious under 35 U.S.C. § 103(a).

Applicants respectfully traverse. To establish a *prima facie* case of obviousness, the Examiner must establish that the prior art reference, or references when combined, render all of the claim limitations obvious: "All words in a claim must be considered in judging the patentability of that claim against the prior art." (*In re Wilson*, 424 F.2d 1382, 165 U.S.P.Q. 494, 496 (CCPA 1970); *see also M.P.E.P. § 2143.03)*. In determining the differences between the prior art and the claims, the question under 35 U.S.C. § 103(a) is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. (*Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 218 USPQ 871 (Fed. Cir. 1983); *Schenck v. Nortron Corp.*, 713 F.2d 782, 218 USPQ 698 (Fed. Cir. 1983), *see also M.P.E.P. § 2143.02)*. Further, there must be a reasonable expectation of success. *In re Merck & Co., Inc.*, 800 F.2d 1091 (Fed. Cir. 1986); *see also M.P.E.P. § 2143.02*. A mere statement that the claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish *prima facie* obviousness. (*M.P.E.P. § 2143.01*). Finally, evidence that rebuts any findings of fact made in support of the rejection under 35 U.S.C. § 103(a), or proof that the claimed invention yields unexpected improved properties can rebut a *prima facie* case obviousness. (*M.P.E.P. §§2141 (V), 2145*).

As set forth in more detail below, the cited references, either alone or in combination, fail to support a *prima facie* case of obviousness, because the cited references and the state of the art

as a whole, would not only fail to lead the skilled artisan to Applicants' presently claimed compositions, but would in fact teach away from the presently claimed embodiments. Further, *even if* the references supported a *prima facie* case of obvious, which Applicants submit is not the case, Applicants demonstration of the unexpected efficacy of the presently claimed embodiments, as well the synergistic effects of the components of the claimed compositions, rebuts any *prima facie* case under 35 U.S.C. § 103(a).

Liu, Dascher, Ravindranath and Lindabald Teach Away From Applicants' Claimed Embodiments

Claims 1, 2, 6, 7, 11, 13-15 and 24-25 are drawn to adjuvants, and vaccines and immunogenic compositions comprising the adjuvant that includes dimethyldioctadecylammonium-bromide, -chloride, -phosphate or –acetate (DDA) and an apolar fraction or part of the apolar fraction of a total lipid extract of a mycobacterium. The teachings of the cited art, as well as the state of the art at the time of filing (as demonstrated by International Patent Application Publication WO 02/074330, cited in the Written Opinion of the International phase of the instant case), would lead the skilled artisan away from selecting either the apolar fraction, or part of the apolar fraction of the total lipid extract of a mycobacterium lipid extract, for combination with DDA in order to produce an adjuvant, as recited in Applicants' present claims.

As an initial matter, contrary to the Examiner's assertion, Liu does not disclose an "adjuvant" that comprises an apolar fraction of a total lipid extract of a mycobacterium, or a part of the apolar fraction of a total lipid extract. Rather, Liu describes the isolation of "nonpeptide antigens" isolated from a lipid extract of *Mycobacterium tuberculosis*. Liu is completely silent regarding an adjuvant that comprises the apolar fraction, or part of the apolar fraction of a total lipid extract of a *Mycobacterium*. Because Liu is concerned with the antigenic properties of *M. tuberculosis* lipids, the skilled artisan would not look to Liu for any guidance relating to adjuvants, and Liu would not provide the skilled artisan with any reasonable expectation that mycobacterial lipids could be used as part of an adjuvant.

Liu teaches that nonpeptide antigens derived from *M. tuberculosis* lipid extracts can be used compositions, such as vaccines. Liu teaches that the lipids of *M. tuberculosis* are immunogenic, and suggests that the antigens can be used to elicit a specific immune response

against the same. Liu teaches that *M. tuberculosis* lipid antigens can be formulated into liposomes, optionally with an adjuvant such as QS-21. Examples 1 and 3 of Liu describe the purification of lipid antigens and formation of vesicles using the same. Example 2 describes the analysis of the immunogenicity of several different lipid antigens. Finally, Example 5 is a prophetic example of a vaccine formulation comprising total lipid extracts together with adjuvant QS-21. There are no data in Liu that relate to the efficacy of the lipid antigens, either as an antigenic component, or as an adjuvant. Therefore, the teachings of Liu cannot provide the skilled artisan with any reasonable expectation that the apolar fraction of total lipid extract from a mycobacterium can be successfully combined with DDA, in order to produce an adjuvant.

Liu teaches that the lipid extract can be fractionated into different polarity classes. In order to elicit an enhanced immune response against *M. tuberculosis*, Liu teaches that it is preferable to include a combination of antigens that comprise lipids from different polarity classes in the vaccine. (See, Liu, at paragraph [0059]). While Liu mentions that *M. tuberculosis* whole lipid extracts can be separated into different polarity classes, there is nothing in Liu that would suggest to the skilled artisan that one should select a particular polarity class of lipids, *e.g.*, the apolar extract or a fraction thereof, as recited in Applicants' present claims, for use in any type of composition, let alone an adjuvant, *e.g.* in combination with DDA. If anything, Liu suggests to the skilled artisan that it is preferable to have a combination of lipid antigens with different polarities in the composition, in order to achieve an enhanced, specific immune response against *M. tuberculosis*. Liu does not mention DDA. As discussed further below, the skilled artisan, at the time of the effective filing date of the instant application, would be led away from combining *M. tuberculosis* lipids with DDA.

The secondary references relied upon by the Examiner in the rejection do not cure the deficiency in Liu, in order to support a *prima facie* case of obviousness. In particular, none of the secondary references cited by the Examiner would lead the skilled artisan to select the apolar fraction, or part of the apolar fraction of a total lipid extract from a mycobacterium and to combine it with DDA. Dascher relates to the use of "whole lipid extracts" from *M. tuberculosis* as antigens in vaccine compositions. As with Liu, the skilled artisan would not look to Dascher for guidance relating to adjuvants, and Dascher would not provide the skilled artisan with any reasonable expectation that lipid extracts can be used as an adjuvant. Dascher is completely

silent regarding the fractionation of the whole lipid extract, and would thus not provide any guidance that would lead the skilled artisan to Applicants' claimed compositions, which recite the apolar fraction, or part of the apolar fraction of a total lipid extract.

Not only is Dascher silent regarding the apolar fraction, or parts of the apolar fraction of mycobacterial lipid extracts, but Dascher provides express teachings that would lead the skilled artisan away from combining lipids with DDA, and therefore would teach away from Applicants' presently claimed embodiments. In particular, Dascher describes the use of lipid antigen vaccination to augment the protective T-cell response to tuberculosis infection. Dascher describes liposome formulations of whole lipid extracts together with QS-21 and/or DDA as adjuvants. The formulations were tested in vaccine experiments for their effects on bacterial burden in guinea pigs. Dascher reports that when DDA alone was used as an adjuvant in combination with whole lipid extracts, there was no significant reduction in bacterial burden. By contrast, formulation of the lipids with QS-21 alone or in combination with other adjuvants did provide a significant reduction in bacterial burden. (Dascher, p. 919, 2nd Col, and Table1). Dascher provides an explanation for the discouraging results with DDA, and suggests that in contrast to QS-21, DDA fails to incorporate into the liposome carrier. The teachings of Dascher would thus lead the skilled artisan to reasonably expect that DDA cannot function with *M. tuberculosis* lipids as such. The teachings of Dascher provide a clear teaching away from Applicants' presently claimed compositions.

As with Liu and Dascher, Ravindranath would not lead the skilled artisan to select the apolar fraction, or part of the apolar fraction of the total lipid extract. In fact, Ravindranath relates to the incorporation of adjuvants into an intracellular compartment, or the outer membrane of, an intact cell, to "ensure[] that the antigens are presented in their natural environment." (Ravindranath, Col. 4., lines 18-19). Ravindranath teaches that extraction of antigens from the cell membrane environment "is likely to alter its immunogenic properties, and thus it is an advantage of the present invention that this is no longer necessary." (Id., Col. 4, lines 23-25). In Table 1, Ravindranath lists over 50 "exemplary adjuvants for conjugation to cells." The list includes whole or part of mycobacterial cells (including phenolic lipids, as asserted by the Examiner), as well as peptides, viruses, nucleic acids, lectins, and the like. However, as with Liu and Dascher, there is nothing in Ravindranath that would reasonably lead the skilled artisan

to select, from the extensive list of adjuvants, the apolar fraction of total lipid extract in combination with DDA. In fact, the Ravindranath list of adjuvants does not even include DDA as one of the numerous exemplary advjuvants. Applicants' presently claimed composition that comprises the apolar fraction, or part of the apolar fraction of total lipid extract from a mycobacterium, in combination with DDA. The numerous adjuvants listed in Ravindranath are allegedly for use in whole-cell vaccine, and are selected as being capable of incorporation into or conjugation to, the membrane of the cells on which the vaccines are based. Not only is Ravindranath completely silent about DDA, but it is also silent about combining any of the numerous adjuvants together. As such, there is nothing in Ravindranath that would suggest to the skilled artisan that one should, or could, combine the apolar fraction, or part of the apolar fraction, of mycobacterial lipid extract with DDA for use as an adjuvant. Accordingly, the skilled artisan would have no reasonable expectation of successfully making an adjuvant from the apolar fraction, or part of the apolar fraction of mycobacterial lipid extract in combination with DDA.

Lindabald also does not cure the deficiencies of the cited references discussed above, in order to establish a *prima facie* case of obviousness. Lindabald describes secreted peptide antigens from *M. tuberculosis*, i.e., short-term culture filtrates or ST-CF in combination with a series of different adjuvants, including DDA. Lindabald teaches that combining ST-CF with DDA produces a modest IFN-γ response in mice which is comparable to ST-CF in combination with saline (no adjuvant), and which is markedly lower than the response to a conventional tuberculosis vaccine, BCG. Lindabald also showed a potent proliferative response in T-cell cultures from mice that had received ST-CF with DDA as an adjuvant, and an antibody response that was strongly skewed to IgG2A (Lindabald, Figure 2). While Lindabald hypothesizes that DDA may be useful as an adjuvant in peptide-based vaccines, Lindabald is completely silent regarding lipid-based vaccines. Given that the teachings of Dascher would lead the skilled artisan away from combining DDA with lipids, the skilled artisan would have no reasonable expectation of successfully forming an adjuvant by combining DDA with lipids, let alone the apolar fraction, or part of the apolar fraction, of the total lipid extract from mycobacterium.

International Patent Application Publication WO 02/074330 confirms the state of the art discussed above, namely that the skilled artisan would have no reasonable expectation of

successfully combining the apolar fraction, or part of the apolar fraction of the total lipid extract of mycobacterium with DDA. Specifically, WO 02/074330 describes the Th1 adjuvant properties of various fractions of mycobacterial lipids. WO 02/074330 teaches that a fraction of the lipids produced from a petrol layer (presumably comprising the apolar lipids), has no activity. The results described in the International Patent Application are consistent with Applicants' own data, demonstrating that apolar lipids are in fact ineffective as adjuvants, unless combined with DDA. (See, e.g., Specification at Table 1).

Applicants also submit herewith as **Exhibit A**, a Declaration under 37 C.F.R. §1.131 by Dr. Dennis Christensen, an expert in vaccine formulation. Dr. Christensen confirms that it was appreciated by those skilled in the art that different classes of adjuvants are commonly used for different classes of vaccines. (Christensen Decl. at ¶5). Further, Dr. Christensen confirms that the state of the art regarding which adjuvants will function well, if at all, in the various types of vaccines was unpredictable. (Id. at ¶5). Finally, according to Dr. Christensen, the state of the art as of the effective filing date of the instant application was such that the skilled artisan would have no reasonable expectation that the apolar lipid fraction of mycobacteria could be used in combination with DDA, since the state of the art, as evidenced by Dascher, suggested that DDA and total mycobacterial lipid extract did not provide immunopotentiating activity that was as good as other adjuvants.

In view of the foregoing, the cited references do not support a *prima facie* case of obviousness. In particular, the combined teachings of the references would lead the skilled artisan away from selecting the apolar fraction or part of the apolar fraction of total lipid extract from mycobacteria and selecting DDA for combination with the apolar lipid extract to make an adjuvant. In view of the lack of guidance and the express teaching away of the combination recited in Applicants' present claims, the skilled artisan would have no reasonable expectation of success, and the specific combination of the apolar fraction of total lipid extract and DDA is not obvious under 35 U.S.C. §103(a).

<u>The Combination of Mycobacterial Lipids and DDA Exhibits Unexpected, Synergistic Efficacy as an Adjuvant</u>

Applicants' presently claimed compositions are based in part on the unexpected discovery that the specific combination of lipid extracts from mycobacterium and DDA provides

unexpectedly beneficial adjuvant properties. As discussed further below, *even if* the cited references supported a *prima facie* case of obviousness under 35 U.S.C. § 103(a), a point which Applicants in no way concede for the several reasons set forth above, the unexpected results observed with Applicants' claimed compositions are sufficient to rebut any *prima facie* showing.

Throughout the specification, Applicants provide data that demonstrate that the specific combination of DDA and the apolar fraction of total mycobacterial lipid extract function together synergistically as an adjuvant, and is significantly more effective when compared to other formulations. In paragraph [0097], Applicants describe experiments demonstrating that antigen combined with DDA alone does not provide significant adjuvant properties, as measured by the release of IFN-γ in response to stimulation with antigen 5 weeks after immunization. (See, Specification, at paragraph [0097] and Figure 5). Similarly, mycobacterial lipids alone do not provide significant adjuvant properties. (Id.). Surprisingly, however, the combination of DDA and mycobacterial lipid extract function synergistically as an adjuvant, as shown by the greater than additive effect of DDA/lipid extract on IFN-γ release. Thus, Applicants discovered that he combination of DDA and mycobacterial lipids function synergistically to provide the beneficial adjuvant properties, which could not have been predicted given the teachings of the art discussed above. The greater than additive effect of DDA/mycobacterial lipids is evidence of the non-obviousness of Applicants' presently claimed compositions. Applicants respectfully submit that this discovery was completely unexpected, particularly in light of the disclosure of Dascher, indicating that DDA does not form proper liposomes when combined with lipid extract.

In paragraphs [0122]-[0126] of the instant specification, Applicants describe experiments that demonstrate the unexpected benefit and efficacy of the apolar fraction of the total lipid extract, when compared to either total lipid extract, or the polar fraction of total lipids. Briefly, apolar fractions, polar fractions, and total lipid extract were prepared from *M. tuberculosis* BCG and formulated with and without DDA. The formulations were used to immunize mice, which were subsequently used in the assays to asses their immune response to *M. tuberculosis* challenge. As shown in Table 7, vaccine formulations using the specific combination of the apolar lipid fraction and DDA as an adjuvant with Ag85B-ESAT elicited about a four-fold more potent immune response compared to DDA combined with total lipid extract, and a five-fold more potent immune response compared to DDA combined with polar lipids, as measured by

IFN-γ release. (Specification, at Table 7). In addition, the apolar lipid fraction alone, or the polar lipid fraction alone did not elicit a significant immune response, thereby confirming the data discussed above showing that the presence of DDA is necessary to achieve the adjuvant effect. The synergistic effect of the combination of the apolar lipid fraction and DDA could not have been predicted. The data showing the five-fold and six-fold increase in potency when compared to DDA/total lipid extract and DDA/polar lipid extract is further evidence of the non-obviousness of Applicants' presently claimed compositions.

Paragraphs [0120]-[0122] of the instant specification describe additional experiments that demonstrate that DDA, when combined with mycobacterial lipids, provides an unexpectedly potent adjuvant effect when compared to different liposomal formulations. In particular, the ability of different liposomal formulations, *i.e.*, DDA, DOTAP, DC-Chol, neutral liposomes, and anionic liposomes, comprising mycobacterial lipids to function as an adjuvant was assessed. As shown in Figure 9, the specific combination of DDA and mycobacterial lipid extracts elicited a much higher immune response (as measured by IFN-γ release), compared to any of the other formulations tested, including DOTAP, another cationic surfactant. Neither neutral liposomes nor anionic liposomes, when combined with mycobacterial lipid extract elicited an immune response. Again, the surprising, synergistic effect of DDA in particular, used in combination with mycobacterial lipids could not have been expected, particularly in light of the state of the art, which taught that the combination of DDA with lipids does not produce a significant immune response.

In addition to the unexpected, significant increase in the immune response described above, Applicants have also discovered that the combination of DDA and mycobacterial lipids beneficially provides for a long-lasting cell mediated immune response (See, e.g., Specification, at paragraph [0032]).

The advantage of formulating a vaccine with DDA and lipids from the apolar class only could not have been foreseen from any of the cited references, particularly in view of Liu, which suggest the benefits of using a combination of lipids with different polarities. The significant increase in efficacy of the specific combination of the apolar lipid fraction and DDA in functioning as an adjuvant could not have been predicted.

Applicants' surprising results relating to the unexpected beneficial effects of the specific combination of DDA and mycobacterial lipids has been confirmed in further studies, *i.e.*, Rosenkrands et al. (2005) Infect. Immun. 73(9): 5817-5826 ("Rosenkrands," submitted herewith as **Exhibit C**). Rosenkrands is an article published after the effective filing date of the instant Application, and co-authored by the inventor of the instant Application. The Christensen Declaration confirms the unexpected effectiveness of the presently claimed compositions shown in the later publication by Rosenkrands.

Rosenkrands examined the immunogenic effect of various vaccines having Ag85B-ESAT6 as the immunogenic component, and different adjuvants. Briefly, Rosenkrands combined total mycobacterial (BCG) lipid extract with DDA, to DOTA, DC-Chol, DOPE, or alhydrogel as an adjuvant, to produce a vaccine with Ag85B-ESAT6 as the antigen. The formulations were used to immunize mice, which were subsequently challenged with BCG and assessed for IFN-γ release and specific antibody responses. The results of the experiments are shown in Figure 2 of Rosenkrands. The level of IFN-γ release in mice immunized with antigen and an adjuvant comprising DDA and total mycobacterial (BCG) lipids, which comprises the apolar fraction, are far superior at stimulating IFN-γ release, compared to any of the other co-adjuvants tested. The DDA and mycobacterial lipids adjuvant also resulted in a considerable antibody response to Ag85B-ESAT6, when compared to DOTA, DC-Chol, DOPE, or alhydrogel.

As explained by Dr. Christensen, Table 2 of Rosenkrands shows that DDA alone is ineffective as adjuvant, and that the effect of using mycobacterial lipids alone is no more than modest (See, Rosekrands, Table 2; Christensen Decl. at ¶12). By contrast, the effect of combining mycobacterial lipids and DDA in cationic liposomes exhibited more than an additive effect when compared to either component alone, demonstrating that the DDA and mycobacterial lipid extract functions synergistically. (See, Rosekrands, Table 1; Christensen Decl. at ¶12). Rosenkrands states that the studies show "*a markedly stronger immune response induced when mycobacterial lipids were administered in combination with cationic liposomes. ...compared to other liposomes, the cationic DDA stood out as the most efficient vehicle in terms of both antibody production and IFN-γ levels induced.*" (page 5824, 2nd column, last paragraph).

Dr. Christensen explains that the data in Rosenkrands also demonstrates superior vaccine protection against infection with *M. tuberculosis* with DDA/mycobacterial lipid extract as an

adjuvant. Table 3 on page 5824 presents results showing vaccine protection against infection with *M. tuberculosis*. The authors show that there was an increased duration of the immune response resulting from DDA based liposomes with mycobacterial lipids (mycosomes), as compared to DDA alone, or DDA combined with MPL (a known immunomodulator).

Finally, Rosenkrands provides data confirming Applicants' discovery that the claimed compositions provide improved long-term resistance compared to other vaccines. Rosenkrands shows that beyond six months after immunization, DDA/mycobacterial lipids/Ag85B-ESAT6 provides significantly higher resistance to *M. tuberculosis* infection, as compared to the *M. tuberculosis* BCG vaccine. (See, Rosenkrands, Fig. 6). Rosenkrands discusses the possible mechanisms responsible for the effects of DDA on duration of protection: Reference is made to earlier studies by Katz et al. 1996 (ref. 22) showing antibody responses of lower duration and more recent studies by Holten-Andersen et al. 2004 (ref. 19) proposing that DDA forms a depot ensuring slow release of antigen. However, Rosenkrands emphasizes that studies with DDA alone never resulted in such striking levels of long-term memory immune responses as seen with the cationic liposomes incorporating apolar mycobacterial lipids, and that the mycobacterial lipids must play an important role: possibly, resistance of the lipids (e.g. phthiocerol dimycoserosates which are part of the apolar fraction) to degradation contribute to the long-term effect (page 5825, 2nd paragraph). The interplay between mycobacterial lipids and DDA could not have been predicted in view of the teachings of the cited art.

As set forth above, the combination of DDA with apolar lipids leads to a profound cellular (IFN-γ) immune response to vaccine antigens as compared to the response to DDA combined with total lipid. (See, Specification, at Table 7). Table 8 shows that on the other hand, that the antibody response to vaccine antigens with DDA/apolar lipid extract as an adjuvant is similar to that observed when DDA/total lipids are used as adjuvant (Table 8). Together, the data demonstrate that the apolar fraction, in combination with DDA causes a re-direction of the immune response toward a cellular response. This re-direction is highly desirable for certain purposes, including tuberculosis vaccination.

The foregoing unexpected effectiveness of the specific combination of DDA and the apolar fraction of mycobacterial lipids provides sufficient evidence to establish that Applicants' presently claimed compositions are not *prima facie* obvious.

The express teaching away of Applicants' presently claimed compositions, coupled with Applicants' discovery of the unexpected profound benefits of the specific combination of components in the presently claimed compositions establish that the compositions of Claims 1, 2, 6, 7, 11, 13-15 and 24-25 would not have been obvious to the skilled artisan. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a).

Rejection Under 35 U.S.C. § 103(a) – Claims 1, 6, 7, 9, 11, 13, 15 and 18-25

The Examiner has rejected Claims 1, 6, 7, 9, 11, 13, 15 and 18-25 as allegedly being unpatentably obvious in view of Liu, in further view of Dascher, U.S. patent Application Publication No. 2002/0176867 to Anderson et al. ("Anderson"), and Lindabald. The Examiner's assertions regarding Liu, Dascher, and Lindabald are discussed above. The Examiner relies upon Anderson as allegedly teaching a tuberculosis vaccine of immunodominant antigens ESAT-6 and Ag85B from *M. tuberculosis*. The Examiner asserts that it would have been obvious to modify the "adjuvant comprising a cationic surfactant an [*sic* – and an] apolar fraction (as disclosed by Liu et al)" to incorporate the antigens ESAT-6 an Ag85B because the use of the ESAT-6 and Ag85B antigens in vaccine compositions was well known in the art, leading to predictable results. (Office Action, at 11).

Applicants respectfully traverse the rejection. Specifically, Anderson is completely silent regarding lipid extracts, and their use in combination with DDA as an adjuvant. As such, Anderson also provides no guidance that would lead the skilled artisan to believe that one could successfully combine the apolar fraction of a mycobacterial lipid extract with DDA to achieve an adjuvant, particularly in view of the teachings expressly against such combination. Accordingly, for at least the same reasons discussed in the rejection of Claims 1, 2, 6, 7, 11, 13-15 and 24-25, Applicants maintain that Claims 1, 6, 7, 9, 11, 13, 15 and 18-25 are not obvious under 35 U.S.C. § 103(a). Applicants respectfully request reconsideration and withdrawal of the rejection accordingly.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of the above amendments and remarks, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: July 16, 2010

By: _____

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EXHIBIT A

PLOUG8.001APC PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Agger, Else.
App. No	:	10/563,731
Filed	:	July 7, 2004
For	:	ADJUVANT COMBINATIONS OF LIPOSOMES AND MYCOBACTERIAL LIPIDS FOR IMMUNIZATION COMPOSITIONS AND VACCINES
Examiner	:	Archie, Nina
Art Unit	:	1645
Conf #	:	1203

DECLARATION UNDER 35 U.S.C. 1.131

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Dennis Christensen declare and state as follows:

1. I am a Research Scientist at Statens Serum Institut, and have extensive experience formulating vaccines. A copy of my curriculum vitae is attached hereto as **Exhibit B**.

2. I am familiar with the above-captioned application, the Office Action dated February 19, 2010, U.S. Patent Application Publication No. 2002/0044951 to Liu et al. ("Liu"), Dascher et al. (2003) *Int. Immunol.* 15(8):915-925 ("Dascher"), U.S. Patent No. 6,218,166 to Ravindranath et al. ("Ravindranath") and Lindabald et al. (1997) *Infect. Immun.* 65(2):623-629 ("Lindabald"), and Rosenkrands, et al., (2005) *Infect. Immun.* 73(9): 5817-5826 ("Rosenkrands").

3. One of the challenges faced in the art of vaccine formulation is the relatively few numbers of adjuvants that are potent, and suitable for human use.

4. There are several different types of vaccines, including whole-cell vaccines, live, attenuated vaccines, subunit vaccines based on peptide antigens, and, more recently, subunit

vaccines based on non-peptide antigens. Vaccines typically have two components: the antigen(s) and the adjuvant(s). Adjuvants are used to non-specifically enhance the immunogenic effect of the antigen with which it is combined.

5. Several adjuvants are known. There are several different classes of adjuvants, including but not limited to polypeptides, nucleic acids, polynucleotides, lipids, sugars, glycolipids, and synthetic compounds. As of July 7, 2004, when the above-captioned application was filed, it was widely accepted by those in the field that different types of adjuvants functioned and were effective with different types of antigens in vaccine formulations. For example, it was generally accepted that a compound that exhibits potent adjuvant effects with one type of antigen (*e.g.*, a nucleic acid-based vaccine), would not necessarily exhibit a potent, or any, adjuvant effect with a different type of antigen (*e.g.*, a polypeptide antigen). It was also appreciated that one could not predict which adjuvants would be particularly effective with other co-adjuvants, or with various antigenic components.

6. The Dascher reference describes mycobacterial lipid vaccines, in which the antigenic component was total lipid extract from *M. tuberculosis*. To formulate the vaccine, the authors used DSPC as carrier lipids, and QS-21 as an adjuvant. The Dascher experiments described on page 919, Col. 2 confirms what was already known in the art regarding mycobacterial lipid antigens, *i.e.* "in the absence of adjuvant, no statistically significant reduction of bacterial colony counts were observed with lipid extracts compared to vehicle control formulation."

7. The Dascher reference also describes experiments in which different adjuvants were combined with the *M. tuberculosis* total lipid extract antigenic components. Briefly, the mycobacterial total lipid extract was combined with the adjuvant QS-21, with or without DDA, or with DDA alone. The animals immunized with the vaccine formulations with QS-21, either with or without DDA, exhibited a significant reduction in bacterial burden after challenge with BCG *M. tuberculosis*. By contrast, the vaccine with the mycobacterial total lipid extract antigenic component and with DDA, a cationic surfactant alone as the adjuvant resulted in "no significant reduction in bacteria" in the immunized animals. (Dascher, at p. 919, Col. 2). Dascher states that the poor activity of DDA alone is likely a result of the failure to incorporate into the liposome carrier. The data in Dascher thus suggest that using DDA as an adjuvant in combination with mycobacterial lipids is undesirable.

8. The data described in the above-captioned patent application and in the Rosenkrands reference show the unexpected efficacy of the combination of DDA/mycobacterial lipids and, in particular DDA/the apolar fraction of mycobacterial lipids – a combination that would not have been expected to function together based upon the data and discussion in the Dascher reference. The data in Table 7 of the specification show that DDA alone does not produce a significant adjuvant effect. The data in Table 7 also confirm that results of Dascher, e.g., that total mycobacterial lipid extract alone does not produce a significant adjuvant effect. The same table shows that the combination of total mycobacterial extract and DDA, however, exhibited a greater than additive adjuvant effect. The synergistic adjuvant effect of the

combination of DDA/ mycobacterial lipids is surprising, and could not have been predicted given the fact that the Dascher discussion suggests that this combination cannot be used to formulate a vaccine. Similar data is also presented in Figure 5 of the above-captioned patent application. Specifically, the data in Figure 5 show that when either DDA alone or total mycobacterial lipid extract alone is used as an adjuvant, there is no significant immune response to challenge. By contrast, the combination of DDA/total mycobacterial lipid extract produced a significant, greater than additive immune response to challenge.

9. In the experiments summarized in Figure 9 of the above-captioned application, various liposomal formulations with mycobacterial lipid extract were assessed for their adjuvant effect. Specifically, mycobacterial lipid extract was combined with DDA, DOTAP, DC-Chol, neutral liposomes an anionic liposomes and an antigenic component. When combined with mycobacterial lipid extract, DDA, in contrast to any of the various other liposomal formulations, is significantly more effective as an adjuvant. The significant effect of DDA, compared to DOTAP, DC-Chol, neutral liposomes an anionic liposomes could not have been predicted.

10. In the experiments summarized in Table 7 of the above-captioned application, different fractions of mycobacterial lipid extract (*i.e.* total extract, the apolar fraction, and the polar fraction) were tested for their adjuvant effect, when combined with DDA. The data in Table 7 demonstrate that the unexpected adjuvant effect of mycobacterial lipid extract/DDA is largely attributable to the apolar lipid fraction. Specifically, Table 7 indicates that the apolar fraction of total lipid extract/DDA is about four-fold more active than total lipid extract/DDA, as measured by stimulation of IFN-γ release. By contrast, the polar lipid fraction of total lipid extract/DDA was less active than the total lipid extract/DDA. The fact that the apolar fraction of the total lipid extract is much more highly active in combination with DDA than either the whole lipid extract or the polar fraction could not have been predicted from any of the references listed in paragraph 1.

11. The Rosenkrands reference confirms the data discussed in paragraph 8, above and further snows that DDA, in contrast to other liposomal formulations, is significantly more effective as an adjuvant when combined with mycobacterial lipid extract. Specifically, in Figure 2 of Rosenkrands shows the relative IFN-γ release in mice immunized with different formulations of the antigen Ag85B-ESAT6 in various adjuvants. Rosenkrands used a combination total mycobacterial (BCG) lipid extract with DDA, DOTAP, DC-Chol, DOPE, or alhydrogel as adjuvants. The level of IFN-γ release in mice immunized with the vaccine in which DDA/total mycobacterial (BCG) lipids was used as the adjuvant, was far superior than compared to the IFN- γ release seen with vaccines using mycobacterial lipid extract in combination with any of the other liposomal formulations. The vaccine with the DDA/mycobacterial lipids adjuvant also caused a considerable antibody response to Ag85B-ESAT6, when compared to the DOTA-, DC-Chol-, DOPE-, or alhydrogel- adjuvants. These data are consistent with the data in the above-captioned patent application, and demonstrate that DDA specifically, when combined with mycobacterial lipids is particularly effective as an adjuvant.

12. Rosenkrands also confirms the synergy between DDA and mycobacterial lipids shown in the above-captioned patent application. Table 2 of Rosenkrands shows that DDA alone is ineffective as an adjuvant, and that the effect of using mycobacterial lipids alone as an adjuvant has only modest activity. However, when DDA is combined with the mycobacterial lipid extract, the adjuvant effect is greater than additive, thereby confirming the synergistic effect of DDA and mycobacterial lipids described in the above-referenced patent application.

13. Rosenkrands demonstrates that the DDA/mycobacterial lipid extract adjuvant provides for unexpectedly superior vaccine protection against *M. tuberculosis* infect. BCG is a standard vaccine against *M. tuberculosis*. Rosenkrands compared the protective effect of a vaccine made of DDA/mycobacterial lipids/Ag85B-ESAT6 to the standard BCG vaccine. The data in Table 3 of Rosenkrands demonstrates that protective effect of the vaccine DDA/mycobacterial lipids/Ag85B-ESAT6 is significantly greater, particularly over time, compared to the standard BCG vaccine.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By:  Date: 14/07-10

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Exhibit B: Curriculum Vitae

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Education/postgraduate work:

June 2010 –	**Head of section**, *Department of Infectious Disease Immunology, Vaccine delivery and formulation,* Statens Serum Institut, Copenhagen/DK www.ssi.dk
Mar. 2010 –	**Centre coordinator,** *Centre for Nano-Vaccines,* www.nano-vaccine.org
June 2008 – May 2010	**Research Scientist**, *Department of Infectious Disease Immunology, Vaccine delivery and formulation,* Statens Serum Institut, Copenhagen/DK www.ssi.dk
May 2005 – May 2008	**Ph.d Student**: Formulation Aspects of the liposomal Adjuvant CAF01 – Freeze-drying and Quantification (*Dissertation handed in 30-04-08, defended 15-08-08*). • *Department of Infectious Disease Immunology, Vaccine delivery and formulation,* Statens Serum Institut, Copenhagen/DK www.ssi.dk • *Faculty of Pharmaceutical Sciences, Institute for Pharmaceutics and Analytical Chemistry, Pharmaceutical Formulation/Biomacromolecules,* University of Copenhagen, Copenhagen/DK www.farma.ku.dk
Aug. 2004 – April 2005	**Scientific associate**, *Department of Infectious Disease Immunology, Vaccine delivery and formulation,* Statens Serum Institut, Copenhagen/DK www.ssi.dk
Feb. 2004 – Aug. 2004	**Master student:** Optimization, analysis and characterization of an adjuvant based on dimethyldioctadecylammonium bromide and Mycobacterium bovis BCG lipids. • *Department of Infectious Disease Immunology, Vaccine delivery and formulation,* Statens Serum Institut, Copenhagen/DK www.ssi.dk • *Danish University of Pharmaceutical Sciences, Institute for Pharmaceutics,* Copenhagen/DK www.farma.ku.dk
Sep. 1998 – Aug. 2004	Matriculated at The Danish University of Pharmaceutical Sciences (DFU)[†].

Postgraduate courses

Aug. 2007 – Nov. 2007	PhD course: **Molecular Biophysics** ((Organizer; SDU, MEMPHYS).
June 2007	PhD course: **Drug Delivery** (Organizer; University of Copenhagen, Faculty of Pharmaceutical Sciences, Dept. Pharmaceutics and Analytical Chemistry).
Nov. 2005	PhD course: **Cellular Molecular Pharmacology and Toxicology** (Organizer; The Danish University of Pharmaceutical Sciences[*], Dept. Pharmacology and Pharmacotherapy)
Nov. 2005	PhD course: **Analytical Challenges in the Formulation Design of Pharmaceutical proteins** (Organizer; The Danish University of Pharmaceutical Sciences[*], Dept. Pharmaceutics and Analytical Chemistry).
Sep. 2005 – Dec. 2005	**Academic Writing in English - Level 1** (Organizer; Dansk Magisterforening)
July 2005	**ULLA Summer School 2005 in Uppasala** (Organizer; *European University Consortium for Pharmaceutical Research*).
April 2005	*Microsoft Access* databases, extended course (Organizer; Assist Danmark)
April 2005	*Microsoft Access* databases, basic course (Organizer; Assist Danmark)
April 2005	Patent protection for Biotechnological Inventions and Pharmaceuticals (Organizer; Chas.Hude Denmark)

[†] Now 'Faculty of Pharmaceutical Sciences, University of Copenhagen'

Nov. 2004 – Feb. 2005	Broad knowledge in existing and coming products from the Vaccine Sector (Internal course at Statens Serum Institut).

Honours:

Received the Thorvald Madsen Prize in 2009, awarded by Statens Serum Institut, Copenhagen, Denmark.

Papers:

Christensen D., T. Lindenstrøm, G. van de Wijdeven, P. Andersen, E.M. Agger. Syringe free vaccination with CAF01 adjuvated Ag85B-ESAT-6 in Bioneedles™ provides strong and prolonged protection against Tuberculosis. 2010, **Submitted**

Henriksen-Lacey M., **D. Christensen,** V.W. Bramwell, T. Lindenstrøm, E.M. Agger, P. Andersen, Y. Perrie; Comparison of the depot effect and immunogenicity of liposomes based on DDA, DC-Chol and DOTAP: Prolonged liposome retention mediates stronger Th1 responses. 2010, **Submitted**

Henriksen-Lacey M., **D. Christensen,** V.W. Bramwell, T. Lindenstrøm, E.M. Agger, P. Andersen, Y. Perrie; Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and immunogenicity of the vaccine. 2010, Accepted, doi:10.1016/j.jconrel.2010.03.027

Henriksen-Lacey M., V.W. Bramwell, **D. Christensen**, E.M. Agger, P. Andersen, Y. Perrie; Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. *Journal of Controlled Release*, 2010, 142 (2) 180-6

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Andersen C.A.S., I. Rosenkrands, A.W. Olsen, P. Nordly, **D. Christensen**, R. Lang, V. Bhowruth, D.E. Minnikin, G.S. Besra, F. Follman, P. Andersen, E.M. Agger; Novel generation mycobacterial adjuvant based on liposome encapsulated monomycolyl glycerol from M. bovis BCG. *The Journal of Immunology*, 2009, 183 (4) 2294-302

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Christensen D., E.M. Agger, L.V. Andreasen, D. Kirby, P. Andersen, Y. Perrie; REVIEW: Liposome based Cationic Adjuvant Formulations (CAF): Past, present & future. *Journal of Liposome Research*, 2009, 19 (1): 2-12.

Agger E.M., I. Rosenkrands, J. Hansen, K. Brahimi, B.S. Vandahl, C. Aagaard, K. Werninghaus, C. Kirschning, R. Lang, **D. Christensen**, M. Theisen, F. Follmann, P. Andersen; Cationic Liposomes Formulated with Synthetic Mycobacterial Cordfactor (CAF01): A Versatile Adjuvant for Vaccines with Different Immunological Requirements. *PLoS ONE* 2008; 3 (9): e3116.

Christensen D., M. Allesø, I. Rosenkrands, J. Rantanten, C. Foged, E.M. Agger, P. Andersen, H.M. Nielsen; NIR transmission spectroscopy for rapid determination of lipid and lyoprotector content in liposomal vaccine adjuvant system CAF01. *Eur J Pharm Biopharm* 2008, 70 (3): 914-20.

Christensen D., D. Kirby, C. Foged, E.M. Agger, P. Andersen, Y. Perrie, H.M. Nielsen; alpha,alpha'-trehalose 6,6'-dibehenate in non-phospholipid-based liposomes enables direct interaction with trehalose, offering stability during freeze-drying. *Biochim Biophys Acta* 2008; 1778: 1365-73.

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Korsholm K.S., E.M. Agger, C. Foged, **D. Christensen**, J. Dietrich, C.S. Andersen, C. Geisler, P. Andersen; The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. *Immunology* 2007; 121(2): 216-26.

Vangala A., V.W. Bramwell, S. McNeil, **D. Christensen**, E.M. Agger, Y. Perrie; Comparison of vesicle based antigen delivery systems for delivery of hepatitis B surface antigen. *Journal of Controlled Release* 2007;119(1): 102-10.

Christensen D., C. Foged, E.M. Agger, I. Rosenkrands og H.M. Nielsen; Frysetørring af vacciner kan redde menneskeliv, Lægemiddelforskning 2006[‡]

Davidsen J., I. Rosenkrands I, **D. Christensen**, A. Vangala, D. Kirby, Y. Perrie, E.M. Agger, P. Andersen; Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6'-dibehenate)-A novel adjuvant inducing both strong CMI and antibody responses. *Biochim Biophys Acta* 2005; **1718**: 22-31.

Book chapters:

Christensen D., K. Korsholm, G.K. Wood, A. Mohammed, V. Bramwell, P. Andersen, E.M. Agger, Y. Perrie; Liposomes in Adjuvant Systems for Parenteral Delivery of Vaccines *in* L. Jorgensen & H.M. Nielsen; *Delivery Technologies for Biopharmaceutics*. Wiley, 2009.

Oral presentations:

Christensen D.; Intratracheal vaccination with Chlamydia vaccine candidates in combination with CAF induces vaginal IgA responses important for protection against Chlamydia trachomatis. Oral presentation at the *Modern Mucosal Vaccines, Adjuvants & Microbiocides,* Dublin, April 2010.

Christensen D.; Vaccine memory and immune quality. Oral presentation at *Vaccines Europe* conference in Brussels, Belgium, November 2009

Christensen D.; Membrane fluidity affects the adjuvant effect of CAF01 liposomes. Oral presentation at *Modern Vaccines - Adjuvants and Delivery Systems* conference in Vienna, Austria, October 2009.

Christensen D.; Vaccine delivery: 'Real world' perspectives: Academic perspective. Oral presentation at *EUFEPS Workshop on Opportunities and Challenges in Vaccine Delivery*, Site d'Archamps, France, September 2008

Christensen D., C. Foged, I. Rosenkrands, E.M. Agger, H.M. Nielsen; Trehalose stabilizes DDA/TDB liposomes via direct interaction during freeze-drying. Oral presentation at the *Day of Research, Faculty of Pharmaceutical Sciences, University of Copenhagen,* Copenhagen/DK, February 2008.

Christensen D., , D. Kirby, C. Foged, E.M. Agger, P. Andersen, Y. Perrie, H.M. Nielsen; Incorporation of TDB enables direct interaction between trehalose and liposomes based on DDA, necessary for stabilization during freeze-drying. Oral presentation at the *ILS meeting 2007*, London/UK.

Christensen D., E.M. Agger; Innate immune responses and adjuvant mechanisms. Oral presentation at the *World Vaccine Congress 2007*, Washington DC

Christensen D., E.M. Agger, I. Rosenkrands, C. Foged, H.M. Nielsen, P. Andersen; Lyophilization of the cationic liposomal adjuvant based on DDA and TDB. Oral presentation at the *Modern Vaccines - Adjuvants and Delivery Systems* conference in London 2006.

Poster presentations[§]:

Christensen D., V. Bramwell, G.K. Wood, Y. Perrie, L. Jørgensen, I. Rosenkrands, P. Andersen , E.M. Agger; Liposomal adjuvant CAF01 and its interaction with protein antigens. Poster at the *Modern Vaccine/Adjuvant Formulation Conference,* Dublin, November 2007.

Christensen D., C. Foged, E.M. Agger, I. Rosenkrands, P. Andersen, H.M. Nielsen; Lyophilization of the cationic liposomal adjuvant based on DDA and TDB. Poster for the International Symposium on Biomolecular Nanoscale Assemblies, *Nano-Science Center, University of Copenhagen*, Copenhagen/DK, January 2007.

Christensen D., E.M. Agger, I. Rosenkrands, C. Foged, H.M. Nielsen; Transport of antigens associated to adjuvant DDA/TDB, across mucosa of an epithelial Calu-3 cell culture model. Poster for the *Day of Research, The Danish University of Pharmaceutical Sciences,* Copenhagen/DK, February 2006.

Christensen D., E.M. Agger, I. Rosenkrands, I. Kromann, P. Andersen; Characterization of a stable adjuvant based on dimethyldioctadecylammonium bromide and trehalose dibehenate. Poster for the *ILS meeting 2005, London.*

Christensen D., I. Rosenkrands, E.M. Agger, C. Foged, H.M. Nielsen; Optimization and characterization of adjuvant formulations based on cationic liposomes and immunomodulating compounds. Poster for the *Drug Research Academy Summer Meeting*, Roskilde August 2005.

[‡] Published by Faculty of Pharmaceutical Sciences, University of Copenhagen
[§] Only posters listed where I was attending the meeting to present it

Christensen D., I. Rosenkrands, E.M. Agger, C. Foged, H.M. Nielsen; Optimization and characterization of adjuvant formulations based on cationic liposomes and immunomodulating compounds. Poster for the *ULLA Summer school*, Uppsala July 2005.

Christensen D., E.M. Agger, I. Rosenkrands, I. Kromann, P. Andersen; Characterization of a stable adjuvant based on dimethyldioctadecylammonium bromide and trehalose dibehenate. Poster for the *Immunopotentiators in Modern Vaccines Conference*, Malaga may 2005.

Andersen, C., **D. Christensen**, E.M. Agger, I. Rosenkrands, J. Davidsen, P. Andersen; Characterization of the adjuvant combination of mycobacterial lipids and dimethylioctadecylammonium bromide liposomes. Poster at the *Modern Vaccine/Adjuvant Formulation Conference,* Prague sep. 2004.

Date: 14/07-10 Signature: 

EXHIBIT C

INFECTION AND IMMUNITY, Sept. 2005, p. 5817–5826 Vol. 73, No. 9
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.9.5817–5826.2005

Cationic Liposomes Containing Mycobacterial Lipids: a New Powerful Th1 Adjuvant System

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The immunostimulation provided by the mycobacterial cell wall has been exploited for many decades, e.g., in Freund's complete adjuvant. Recently, the underlying mechanism behind this adjuvant activity, including Toll receptor signaling, has begun to be unraveled, confirming the potential of mycobacterial constituents to act as adjuvants. In this study, the immunostimulatory properties of a *Mycobacterium bovis* BCG lipid extract were tested for their adjuvant activity. Administration of the lipids in dimethyl dioctadecyl ammonium bromide-based cationic liposomes induced a powerful Th1 response characterized by markedly elevated antigen-specific immunoglobulin G2a (IgG2a) isotype antibodies and substantial production of gamma interferon. The adjuvant formulation (designated mycosomes) elicited high levels of gamma interferon both in C57BL/6 as well as in Th2-prone BALB/c mice. Furthermore, the mycosomes induced immune responses to protein antigens from several sources including *Mycobacterium tuberculosis*, *Chlamydia muridarum*, and tetanus toxoid. In a tuberculosis challenge model, the mycosomes combined with the Ag85B-ESAT-6 fusion protein were demonstrated to have a unique ability to maintain sustained immunological memory at a level superior to live BCG.

Vaccine research in recent years, both in the infectious disease and cancer fields, has highlighted the need for effective adjuvant formulations that induce cellular immune responses. The need for adequate adjuvants applies not only to vaccines based on recombinant proteins and synthetic peptides of inherently low immunogenicity but also to coadministration with other constructs such as DNA vaccines and adenoviral vectors (29, 42). Despite the general need for robust adjuvants and delivery systems for modern vaccines, the only adjuvants approved for human use worldwide are, even today, Th2-promoting aluminum-based compounds. This has led to a major interest in the development of Th1-inducing adjuvants for human use. Although a number of candidates have been evaluated (32), a Th1 adjuvant for human use has yet to be approved.

Improved understanding of the initiation of immune responses and, in particular, the discovery of receptors recognizing microbial constituents, has revealed a new strategy for adjuvant research. By mimicking these pathogen-associated molecular patterns, it is now possible to design synthetic analogues that act as ligands for these receptors. In this context, a panel of synthetic lipid A derivatives has been designed to serve as agonists for human Toll-like receptor 4 (TLR4) (6), while other well-known adjuvants, such as unmethylated CpG DNA motifs, have been found to be ligands for TLR9. However, given the complexity of the events leading to a protective immune response, the new generation of adjuvants is unlikely to be based on a single component. Rather, more complex adjuvant formulations based on combinations of several monotherapeutic agents capable of targeting multiple different receptors, and therefore more likely to induce complex and sufficient immune responses, are seen as the way forward (31). One example of this development is represented by the development of the archaeosomes based on the polar lipid fraction from archaea. The archaeosome adjuvant system has been characterized in detail by Sprott and coworkers, and it is able to induce a humoral as well as a cell-mediated immune response (25).

Preparations of mycobacteria have been shown to exert their effect by signaling through several TLRs (8) and have long been recognized as an important source of immunostimulants. We have investigated the immunostimulatory capacity of a total lipid extract of *Mycobacterium bovis* BCG obtained using a simple purification process suited for large-scale production. The lipid extract, delivered in cationic liposomes based on dimethyl dioctadecyl ammonium bromide (DDA) and designated mycosomes, was found to generate strong antigen-specific immune responses to a range of different antigens. This immune response was characterized by high levels of antibodies and gamma interferon (IFN-γ) and a very efficient maintenance of immunological memory.

MATERIALS AND METHODS

Reagents. DDA was obtained from Eastman Kodak, Inc. (Rochester, NY). Phosphatidylglycerol (PG), L-phosphatidylcholine (PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTAP), and cholesteryl 3b-*N*-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol) were all purchased from Sigma Aldrich Denmark (Brøndby, Denmark). Alum (2% alhydrogel) was from Brenntag Biosector (Frederikssund, Denmark), and lipid A was from Avanti Polar Lipids (Alabama).

Extraction of lipids. *M. bovis* BCG was cultured in modified Sauton medium (2). The mycobacteria were harvested after 2 to 3 weeks of culturing, suspended

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† I.R. and E.A.G. contributed equally to this work.

TABLE 1. Overview of the subunit vaccines used for immunization of mice

Antigen	Adjuvant
	100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DDA
2 μg of Ag85B-ESAT-6	100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	None
10 μg of MOMP	None
10 μg of ovalbumin	None
10 μg of tetanus toxoid	None
2 μg of Ag85B-ESAT-6	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of MOMP	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of ovalbumin	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
10 μg of tetanus toxoid	Mycosomes (250 μg of DDA/100 μg of BCG lipids)
2 μg of Ag85B-ESAT-6	250 μg of DDA/25 μg of MPL
2 μg of Ag85B-ESAT-6	250 μg of DOTAP/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DC-Chol/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DOPE/PC/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	250 μg of DOPE/PC/PG/100 μg of BCG lipids
2 μg of Ag85B-ESAT-6	500 μg of alum

in phosphate-buffered saline (PBS), and incubated for 1.5 h hours at 60°C. After centrifugation and removal of the supernatant, lipids were extracted by treating 10 to 15 g of mycobacteria (wet weight) with 30 ml of chloroform-methanol (2:1) for 15 min at 55°C. The extraction was repeated, and the organic phases from both extractions were pooled and washed twice with 5 ml of water to remove hydrophilic molecules. The solvent of the organic phases was evaporated, and the amount of dry lipid material was weighed, redissolved in chloroform, and aliquoted into vials of 1 or 5 mg, followed by evaporation of chloroform and storage at −20°C.

Antigens. The fusion protein of Ag85B and ESAT-6 (hereafter designated Ag85B-ESAT-6) was produced as a recombinant protein as previously described (33). Ovalbumin was obtained from Sigma; tetanus toxoid was from Statens Serum Institut, Copenhagen, Denmark. The recombinant major outer membrane protein (MOMP) from *Chlamydia muridarum* was expressed in the pDest17 system (Gateway; Invitrogen) and purified as previously described (40).

Adjuvants and vaccines. Total lipid extracts were prepared by rehydrating dry *M. bovis* BCG lipid material with Milli Q water at 1 or 5 mg/ml, followed by probe sonication on a Sanyo Soniprep 150 MSE sonicator (2 pulses of 30 s at amplitude of 10 μm).

DDA was prepared by adding DDA powder to sterile distilled water (2.5 mg/ml) and heating at 80°C under continuous stirring for 20 min, followed by cooling to room temperature before use. The standard mycosome vaccine was prepared by mixing the antigen with saline, followed by the addition of rehydrated lipid extract and DDA and vortex mixing. The vaccine was left overnight to allow adsorption of the antigen.

Other liposomes were composed of DOTAP, DC-Chol, PC-DOPE (neutral liposomes; molar ratio of 1:0.5) or PC-DOPE-PG (anionic liposomes; molar ratio of 1:0.5:0.25). Vaccines for a total of five mice were prepared by evaporation of solvent from 1.25 g of the total liposome-forming compound(s) dissolved in chloroform. The dry lipid material was hydrated with 500 μl of Milli Q water and sonicated for 30 min in a bath-type sonicator. Ten micrograms of antigen in 100 μl of 50 mM ammoniumcarbonate buffer and 500 μl of BCG lipids (1 mg/ml) were added, followed by lyophilization. The lipid-antigen mixture was rehydrated by the addition of 1,000 μl of saline.

Alum was added to the antigen mixed with saline immediately before immunization. DDA-monophosphoryl lipid A (MPL) was prepared as previously described (10). An overview of the various adjuvant preparations used in this study is provided in Table 1.

Characterization of BCG total lipid extract. Two-dimensional thin layer chromatography (2D-TLC) of *M. bovis* BCG lipid extracts was performed by Claire Reid at the Scottish Crop Research Institute according to the method of Dobson et al. (13). A total of 1.4 mg of lipid material dissolved in chloroform-methanol (2:1) was applied for each analysis. An *M. tuberculosis* standard lipid extract prepared and characterized as previously described (13) was used as a standard for the TLC.

Apolar lipids were analyzed in the following system: first direction, petroleum ether (bp 40 to 60°C)-ethyl acetate (98:2); second direction, petroleum ether (bp 40 to 60°C)-acetone (98:2). Nonpolar lipids were detected with 20% molybdophosphoric acid in ethanol and heated at 120°C.

Polar lipids were analyzed in the following system: first direction, chloroform-methanol-water (60:30:6); second direction, chloroform-acetone-methanol-water (47:25:3:5). Polar lipids were detected with 20% molybdophosphoric acid in ethanol and heated at 120°C; ninhydrin reagent was used to detect lipids with free amino groups, and Phospray (Sigma) was used to detect phospholipids.

Glycolipids of intermediate polarity were analyzed in the following system: first direction, chloroform-methanol-water (100:14:0.8); second direction, chloroform-acetone-methanol-water (50:60:2.5:3). Glycolipids were detected by α-naphthol reagent and heating at 110°C.

Ten-microliter samples of rehydrated lipid extracts (1 mg/ml) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and silver staining (9) for residual protein content.

The adsorption of antigen to mycosomes was investigated by mixing 50 μg of antigen and 0.5 mg of rehydrated lipid extract prepared as described above with 1.25 mg of DDA in a total volume of 1 ml. Adsorption was allowed to proceed overnight. The samples were then ultracentrifuged (100,000 × g for 1 h). The supernatant was collected and the pellet was resuspended in the original volume (1 ml); both samples were analyzed by SDS-PAGE and silver staining. Protein concentrations in the supernatants were determined by the microbicinchoninic acid method according to the manufacturer's instructions (Pierce Europe, Oud-Beijerland, The Netherlands), and the amount of antigen adsorbed to the mycosomes was determined as previously described for DDA-BBG2Na solutions (23).

Particle size analysis was performed by photon correlation spectroscopy using a Malvern Zetasizer 4 with a ZET 5110 cell (Malvern Instruments, Ltd., Worcestershire, United Kingdom). The *Z*-average diameter and the polydispersity index (PI) were determined. Small values of PI (<0.1) indicate a population of low heterogeneity, while PI values of >0.3 indicate high heterogeneity.

A test for pyrogenicity in the standard rabbit model was performed by Charles River (Wiga, Germany) according to the European Pharmacopoeia. Rabbits were given an intravenous dose (0.5 ml/kg of body weight) of different concentrations of mycosomes, and rectal temperatures were recorded for 3 h after administration.

Animals. Female BALB/c or C57BL/6 mice, 8 to 12 weeks old, were obtained from Bomholtgaard (Ry, Denmark) or Harlan Scandinavia (Denmark). Infected mice were kept in cages within a BL-3 laminar flow safety enclosure.

Immunization. Mice were immunized subcutaneously (s.c.) with vaccines containing 0 μg (adjuvant controls), 2 μg (Ag85B-ESAT-6), or 10 μg of the antigen (all other antigens) in a total volume of 0.2 ml, at the base of the tails three times with a 2-week interval between each immunization. As a positive control in the experiment involving *M. tuberculosis* infection, a single group of mice received one dose of BCG Danish 1331, 5×10^6 CFU, injected s.c. at the base of the tail.

Determination of antibody titers. Plates for enzyme-linked immunosorbent assay (Nunc maxisorp, Roskilde, Denmark) were coated with ovalbumin (2 μg/well), Ag85B-ESAT-6, MOMP, or tetanus toxoid (0.05 μg/well) in PBS overnight at 4°C. Free binding sites were blocked with PBS containing 2% skim milk. Individual mouse serum from three to four mice per group was analyzed in duplicate in fivefold dilutions at least 10 times in PBS with 1% bovine serum albumin; the initial dilution was 20-fold. After a washing procedure, horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-mouse immunoglobulin G1 [IgG1] and IgG2a; Zymed) diluted 1/2000 in PBS containing 1% bovine serum albumin was added. Following 1 h of incubation, antigen-specific antibodies were detected by TMB (3,3′,5,5′-tetramethylbenzidine) substrate as described by the manufacturer (Kem-En-Tec, Copenhagen, Denmark). The absorbance values were plotted as a function of the reciprocal dilution of serum samples. The data were fitted by nonlinear regression analysis with a sigmoidal dose-response curve of variable slope by the GraphPad Prism program (version 4.00; GraphPad Software Inc.). Antibody titers were then defined as the serum dilution that gives an absorbance value of 1.00 in the parallel portion of the curves (39). For serum samples where the antibody titration curves were below this value, the titer was defined as below the dilution 20 (<20).

Cellular assays. Blood samples were drawn from mice 7 days after the last immunization, pooled from five to six mice in each group, and the blood lymphocytes were obtained (3). Splenocytes were isolated from mice 7 days after the last immunization as previously described (3). Cell cultures were performed in

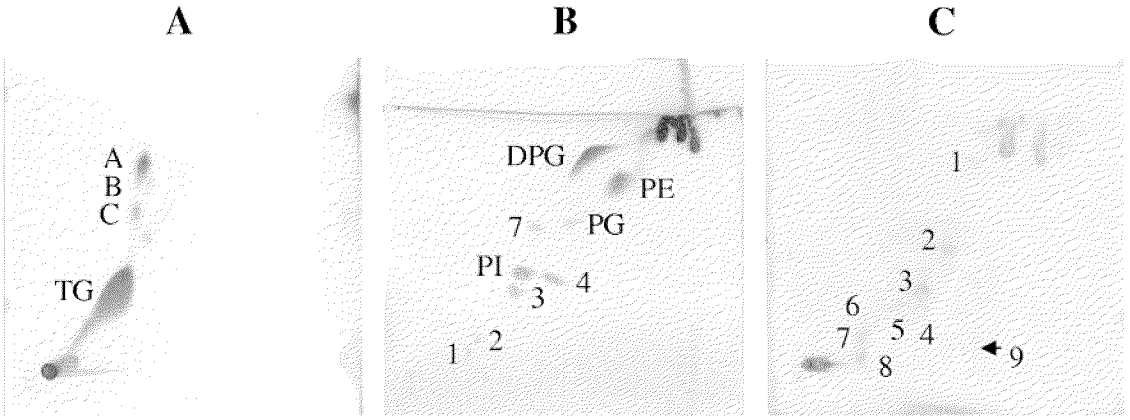

FIG. 1. 2D-TLC analysis of BCG lipids extract. Apolar (A), polar (B), and glycolipids of intermediate polarity (C) were detected. In the apolar fraction (panel A), triacylglycerol (TG) and phthiocerol dimycocerosate A, B, and C (A, B, and C) were detected. The polar fraction in panel B contained phosphatidylinositol mannosides (1–4), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and L-alpha-phosphatidyl-DL-glycerol (PG). In the fraction of intermediate polarity shown in panel C, the numbers 1 to 9 indicate glycolipids of intermediate polarity that were not identified.

triplicate in round-bottomed microtiter wells containing 2×10^5 cells in a volume of 200 μl RPMI medium supplemented with 2-mercaptoethanol, glutamine, penicillin-streptomycin, HEPES, and 10% fetal calf serum. Antigens were used in concentrations ranging from 5 to 0.08 μg/ml. Wells containing medium only or 5 μg/ml of concanavalin A were included in all experiments as negative and positive controls, respectively. Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of antigen, and the amount of IFN-γ was determined by enzyme-linked immunosorbent assay using purified rat anti-mouse IFN-γ (BD Pharmingen, San Diego, CA) as the coating antibody, biotin-labeled rat anti-mouse IFN-γ (BD Pharmingen) as capture antibody, and HRP-conjugated streptavidin (Zymed, San Francisco, CA) for detection of IFN-γ. The presence of interleukin-5 (IL-5) was analyzed similarly using anti-mouse IL-5 coating and capture antibodies (BD Pharmingen) and HRP-conjugated streptavidin (Zymed).

To evaluate the responding T-cell subset, the CD4 and CD8 T-cell receptors were blocked as previously described (1).

Fluorescence-activated cell sorting analysis. Splenocytes were isolated from mice 7 days after the last immunization and restimulated in 96-well U-bottom plates containing 5 μg/ml of Ag85B-ESAT-6 and 2×10^6 cells/well. Control wells without antigen were also included. After restimulation overnight, brefeldin A (Sigma) was added to a final concentration of 2.25 μg/well, and the cultures were further incubated for 4 h. After cells were washed, nonspecific binding was blocked by a 15-min incubation with the 24G2 clone (CD16/CD32; BD Pharmingen) and subsequently stained with peridinin chlorophyll protein-CD4 and allophycocyanin-CD8 (both BD Pharmingen) on ice for 20 min. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit available from BD Pharmingen according to the manufacturer's protocol and using phycoerythrin–IFN-γ (BD Pharmingen). Cells were finally washed three times, resuspended in paraformaldehyde, and analyzed with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA) by collecting 50,000 events.

Experimental infections. For evaluation of vaccine efficacy, mice were challenged 2.5, 6, or 14 months after the first immunization by the aerosol route in a Glas-Col inhalation exposure system (Inhalation Exposure System 099C A4224; Glas-Col, IN) calibrated to deposit approximately 25 CFU of virulent *M. tuberculosis* Erdman in the lungs. The bacterial loads in spleen and lungs were determined 6 weeks later by plating serial dilutions onto Middlebrook 7H11 agar supplemented with 2 μl of 2-thiphene-carboxylic acid hydrazide per ml to selectively inhibit the growth of BCG. Colonies were counted after 2 to 3 weeks of incubation at 37°C.

Statistical analyses. Differences in numbers of colonies between infected mice and control mice were tested by analysis of variance. When significant effects were indicated, differences between means were assessed by a Dunnetts test.

RESULTS

Preparation and characterization of a mycobacterial total lipid extract. *M. bovis* BCG was chosen as the starting material for the preparation of a mycobacterial lipid extract obtained using a standard chloroform-methanol extraction method. The total lipid composition of the extract was analyzed by 2D-TLC for apolar and polar lipids and lipids of intermediate polarity, according to the method outlined by Dobson et al. (13). Of the apolar lipids present, phthiocerol dimycocerosates A, B, and C and triacylglycerol were identified (Fig. 1A). The polar lipids identified were phosphatidylinositol mannosides, phosphatidylinositol, phosphatidylethanolamine, and diphosphatidylglycerol (Fig. 1B). Small amounts of L-alpha-phosphatidyl-DL-glycerol sodium salt and an unknown phospholipid were also detected. Nine glycolipids of intermediate polarity were detected in the lipid extract. These lipids were not identified (Fig. 1C). Comparison of the 2D-TLCs obtained for three independent extractions of BCG lipids showed the same overall TLC profiles (data not shown).

The lipid extract was subsequently tested for protein contamination by silver-stained SDS-PAGE. The presence of protein bands was not detected even after extensive development of the gel (data not shown). Before the lipid extract was used for immunization, the pyrogenicity of the lipids was tested in an established rabbit pyrogenicity model. The results from this model demonstrated that the lipids were not pyrogenic even at concentrations up to 1.0 mg/ml (results not shown).

Liposomes as vehicles for mycobacterial lipids. A range of different cationic, neutral, and anionic lipid formulations have previously been used as vehicles for immunomodulators to obtain efficient adjuvant systems. Therefore, the ability of BCG lipids to modulate immune responses when delivered entrapped in liposomes of different charges was investigated. In this study, the tuberculosis (TB) subunit candidate, Ag85B-ESAT-6, was used as a model antigen. Ag85B-ESAT-6 was administered in combination with the BCG lipids in cationic liposomes formed of either DDA, DOTAP, or DC-Chol; neutral liposomes formed by DOPE-PC; and anionic liposomes formed by DOPE-PC-PG. For comparison, Ag85B-ESAT-6 was also administered in the traditional adjuvant, alum. Immune responses were monitored by in vitro restimulation of peripheral blood mononuclear cells purified 1 week after the

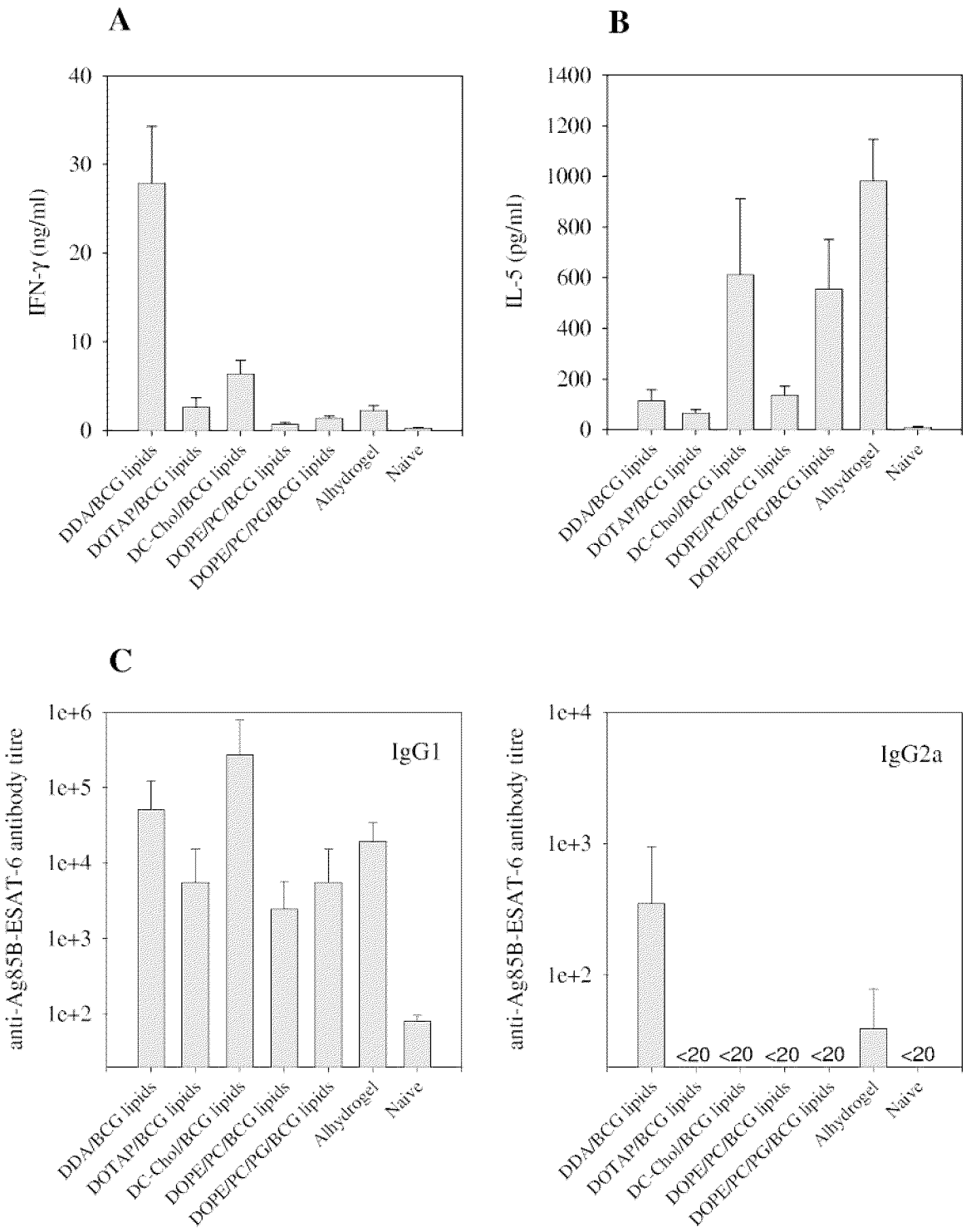

FIG. 2. Immune responses generated by BCG lipids entrapped in liposomes of different charges. Release of IFN-γ (A) or IL-5 (B) from blood lymphocytes isolated from BALB/c mice immunized with 2 μg of Ag85B-ESAT-6 in DDA-BCG lipids, DOTAP-BCG lipids, DC-Chol-BCG lipids, DOPE-PC-BCG lipids, DOPE-PC-PG-BCG lipids, or naïve mice. Blood lymphocytes were isolated 5 weeks after the first immunization and restimulated in vitro with Ag85B-ESAT-6 (5 μg/ml). (C) Antigen-specific antibody midpoint titers in serum from BALB/c mice immunized with Ag85B-ESAT-6 measured as IgG1 and IgG2a titers.

last immunization. As shown in Fig. 2A, BCG lipids together with DDA elicited the most pronounced levels of IFN-γ release. In contrast, IL-5 production was mainly seen in mice immunized with alum, DC-Chol, or DOPE-PC-PG, while DDA-BCG lipids only gave minimal levels of IL-5 (Fig. 2B). Analysis of antigen-specific antibodies demonstrated an efficient induction of both IgG1 and IgG2a antibodies by the combination of DDA-BCG lipids (Fig. 2C). The IgG1 titer

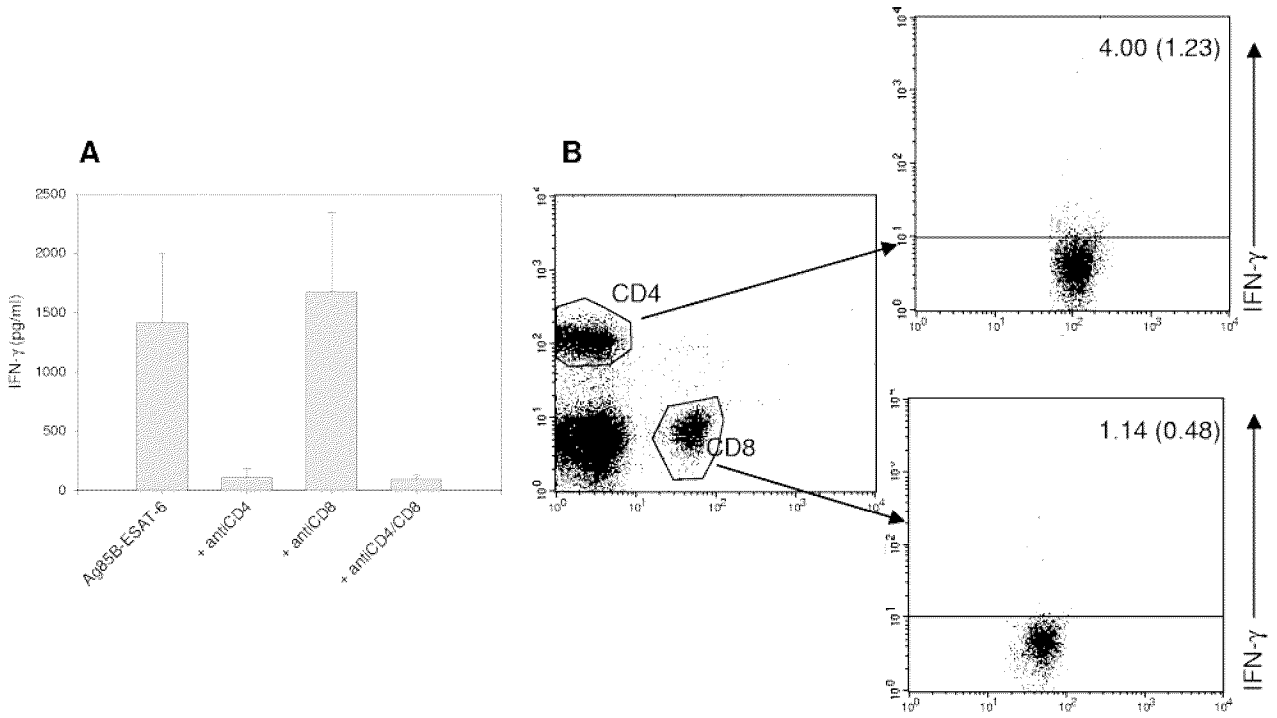

FIG. 3. Evaluation of the IFN-γ responses of CD4 and CD8 T-cell subsets. (A) Release of IFN-γ from spleen lymphocytes isolated from BALB/c mice immunized with 2 μg of Ag85B-ESAT-6 in mycosomes. Splenocytes were isolated 1 week after the first immunization and blocked with anti-CD4, anti-CD8, or anti-CD4/CD8 prior to restimulation with 5 μg/ml of Ag85B-ESAT-6. (B) Percentage of the CD4 and CD8 T-cell population positive for IFN-γ in the spleen of immunized mice. The percentage of IFN-γ positive cells in the spleens of nonimmunized control mice are indicated in the parentheses. The splenocytes were restimulated with Ag85B-ESAT-6 overnight. The graph is representative of three individual mice.

obtained with this combination was, in fact, 2.6-fold higher than when the antigen was administered in alum. Furthermore, although the IgG1 antibody titers were higher than the IgG2a titers, the ratio of IgG2a:IgG1 was clearly increased after administration of DDA-BCG lipids compared to both alum and the other combinations of liposomes and BCG lipids. Together, these results demonstrate the induction of an efficient Th1 cell-mediated immune response with the DDA-BCG lipids, and this combination (in the following referred to as mycosomes) was therefore subjected to further investigation. The immunological response was compared for three independent batches of BCG lipids, and the same level of IFN-γ release was obtained for the extracts (results not shown). In order to optimize the mycosomes, different amounts of lipids were tested together with the standard dose of 250 μg of DDA per mouse. These studies demonstrated an optimum dose of 100 μg of BCG lipids (results not shown).

For evaluation of the T-cell subset mediating the response observed with mycosomes, spleen cultures from immunized mice were blocked with monoclonal antibodies against the CD4 and CD8 receptors prior to restimulation with Ag85B-ESAT-6. As shown in Fig. 3A, the response was completely abrogated by anti-CD4, whereas no effect was observed by blocking CD8 T cells. Flow cytometry analysis of T cells and intracellular IFN-γ staining provided further evidence that the CD4 T cells are the responding subset in mycosome-immunized mice (Fig. 3B).

Mycosomes induce Th1 responses in C57BL/6 and BALB/c mice. DDA has previously been used in combination with immunomodulators such as MPL for enhancing their inherent adjuvant activity (10). For comparison, DDA-MPL as well as mycosomes were used as adjuvant formulations in two mice strains of different genetic backgrounds (11). Groups of C57BL/6 and BALB/c mice were immunized three times at 14-day intervals with 2 μg of Ag85B-ESAT-6, and the immune responses induced were assessed 5 weeks after the first vaccination. In C57BL/6 mice, both adjuvant combinations induced strong IFN-γ responses after restimulation with the vaccine antigen (Table 2). In BALB/c mice, in contrast, only the mycosomes induced a strong Th1 recall response to the Ag85B-ESAT-6 antigen with high levels of IFN-γ. DDA-MPL, in comparison, induced much more modest levels of IFN-γ in this Th2-biased mouse strain. The lipid extract exhibited no activity alone, emphasizing the necessity for a vehicle to maximize the immunostimulatory effects of the lipids.

Characterization of the mycosomes. Particle size analysis of DDA alone gave a *Z*-average size of 853 nm and a polydispersity index of 0.198, while the rehydrated BCG lipids showed a *Z*-average diameter of 290 nm and a polydispersity index of 0.553. The mycosomes gave a *Z*-average diameter of 738 nm and a polydispersity index of 0.300, indicating that a more homogenous particle size distribution is obtained compared to the BCG lipids on their own.

The amount of either Ag85B-ESAT-6 or ovalbumin ad-

TABLE 2. Ag85B-ESAT-6 responses in mice vaccinated with different adjuvant combinations

Vaccine[a]	IFN-γ (ng/ml) ± SD[b]	
	C57BL/6	BALB/c
Expt 1		
DDA	6.27 ± 2.93	ND
DDA/MPL	23.6 ± 7.50	0.40 ± 0.05
Mycosomes	118.2 ± 23.8	11.0 ± 0.10
Expt 2		
DDA	1.60 ± 0.40	ND
BCG lipids	0.06 ± 0.04	ND
Mycosomes	19.29 ± 0.29	ND

[a] Mice were immunized three times with 2 μg of Ag85B-ESAT-6 in the indicated adjuvant (n = 3 to 6 mice).

[b] Peripheral blood mononuclear cells were purified from the blood one week post the last immunization and restimulated with 5 μg/ml of Ag85B-ESAT-6. The presence of IFN-γ was measured in the supernatants using ELISA. ND not done.

sorbed to the mycosomes was analyzed by SDS-PAGE and silver staining after ultracentrifugation. The majority of the antigen was found in the adjuvant pellet of the vaccine, indicating a very efficient adsorption of both antigens to the mycosomes (Fig. 4). As a control, ultracentrifugation of the antigen solution alone demonstrated minimal precipitation or aggregation as the vast majority of the antigen was found in the supernatant (data not shown). Quantitation of the amount of protein in the supernatant by the microbicinchoninic protein assay showed that 89 and 88% of ovalbumin and Ag85B-ESAT-6, respectively, were found to be adsorbed to the mycosomes.

Mycosomes as adjuvants for different antigens. In this study, Ag85B-ESAT-6 was used in all the initial studies of immune responses promoted by mycosomes; we continued by comparing the adjuvant activity for a panel of nonmycobacterial antigens, namely, the MOMPs (major outer membrane proteins) from *C. muridarum*, tetanus toxoid, and ovalbumin. All antigens were administered together with our standard dose of mycosomes three times by the s.c. route, and immune responses were monitored 5 weeks after the first immunization.

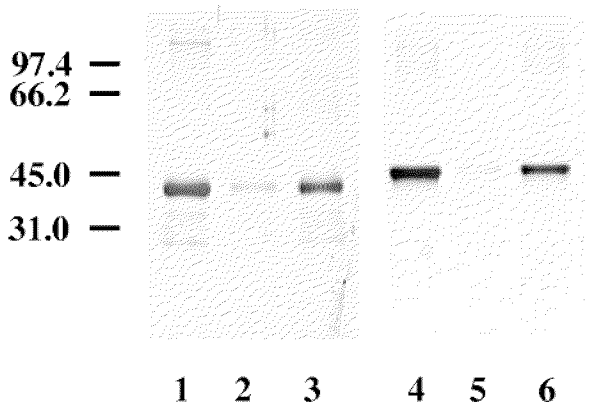

FIG. 4. Silver-stained SDS-PAGE gel of antigen adsorption to mycosomes. Lane 1, Ag85B-ESAT-6; lane 2, Ag85B-ESAT-6 in mycosome supernatant; lane 3, Ag85B-ESAT-6 in mycosome pellet; lane 4, ovalbumin; lane 5, ovalbumin in mycosome supernatant; lane 6, ovalbumin in mycosome pellet.

High levels of IFN-γ were recorded after immunization with MOMP and tetanus toxoid, whereas administration of ovalbumin in mycosomes gave rise to only modest levels of IFN-γ (Fig. 5). Both ovalbumin and the rest of the panel gave rise to high levels of IgG1 antibodies after immunization with the individual antigens. The IgG2a titers to each antigen were lower than the IgG1 titers but in agreement with the high IFN-γ levels; MOMP gave rise to the highest increase of IgG2a. Compared to administration of the antigens without adjuvant, the observed increase in IgG1 titers (n-fold) for MOMP, ovalbumin, and tetanus toxoid were 4.2, 970, and 47, respectively. For IgG2a, the observed increase in titers (n-fold) for MOMP, ovalbumin, and tetanus toxoid were 16, 4.2, and 5.2, respectively. These findings demonstrate that the mycosomes can be used to enhance immune responses to proteins with different characteristics and from sources other than *M. tuberculosis*.

Protective efficacy of the Ag85B-ESAT-6 delivered in mycosomes. In order to evaluate the actual efficacy of the Ag85B-ESAT-6 delivered in mycosomes against an *M. tuberculosis* challenge infection, BALB/c mice were immunized three times with Ag85B-ESAT-6 emulsified in mycosomes as well as the single components and challenged with virulent *M. tuberculosis* Erdman by the aerosol route 10 weeks after the first immunization. As shown in Table 3, Ag85B-ESAT-6 emulsified in mycosomes induced high levels of protection in both spleen and lungs, whereas a more modest efficacy was obtained using BCG lipids alone to emulsify Ag85B-ESAT-6. No significant protection was found in groups receiving the antigen in DDA or with antigen or BCG lipids alone. Furthermore, nonsignificant levels of protection have been observed with the mycosome preparation alone with a $\log_{10}$ reduction of 0.0 to 0.2 compared to nonimmunized mice (results not shown).

Efficient maintenance of immunological memory by mycosomes. One of the most important features for any new adjuvant system is the maintenance of immunological memory. We assessed the longevity of immunity by measuring responses in the blood and spleen at various time points postimmunization (results not shown). After an initial peak of IFN-γ release at 3 weeks after the final immunization, the levels declined somewhat. However, high levels were still observed as late as 14 months postimmunization (8,953 ± 41 and 1,796 ± 299 pg/ml in the blood and spleen, respectively). The responding cells had a classical effector phenotype characterized by high expression of CD44 and low expression of CD62L (results not shown). Subsequently, the mice were given an aerosol challenge at 2.5 months, 6 months, and 14 months after the first immunization, and the number of CFU in the lungs was monitored. The protection promoted was compared to a standard BCG vaccine. Although BCG gave rise to the highest level of protection at the earliest time point, Ag85B-ESAT-6 emulsified in mycosomes gave rise to high and significant levels of protection at all time points (Fig. 6). Moreover, whereas the protection level of the live BCG vaccine waned over time, the protective efficacy of Ag85B-ESAT-6 administered in mycosomes increased throughout the study period and gave rise to a significantly higher level of protection compared to BCG at the late time point ($P < 0.01$).

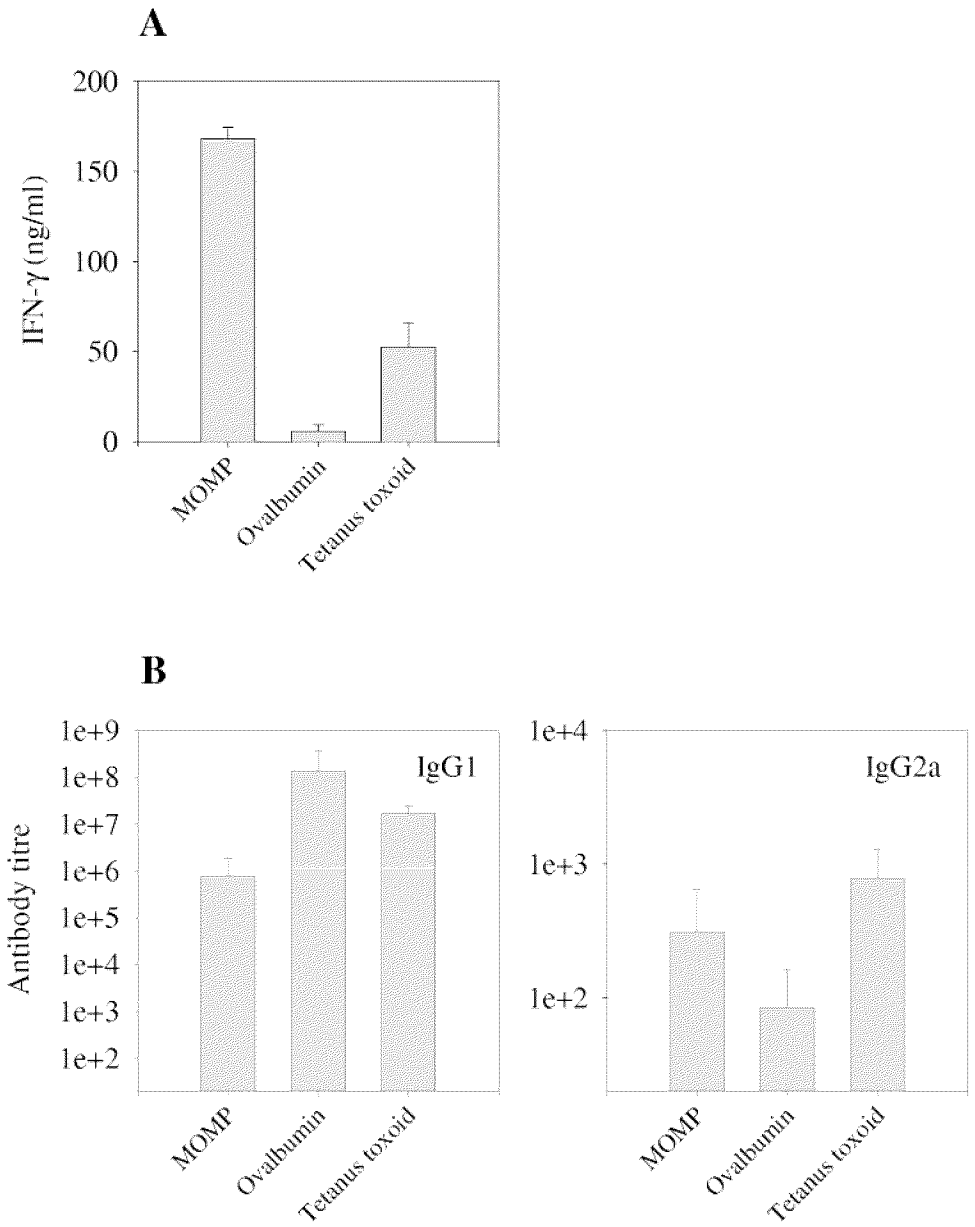

FIG. 5. Induction of immune response using mycosomes as adjuvant for various antigens. (A) Release of IFN-γ from blood lymphocytes isolated from BALB/c mice immunized with 10 μg of MOMP, ovalbumin, or tetanus toxoid in mycosomes. Blood lymphocytes were isolated 5 weeks after the first immunization and restimulated in vitro with the antigen used for immunization (5 μg/ml). (B) Antigen-specific antibody midpoint titers in serum from immunized BALB/c mice measured as IgG1 and IgG2a titers. All results have been compared to naïve mice.

DISCUSSION

Herein, it is demonstrated that a liposomal formulation of mycobacterial lipids is capable of inducing strong humoral as well as cell-mediated immune responses against both mycobacterial and nonmycobacterial antigens. The mycobacterial lipid extract consists of several lipids as demonstrated by TLC and will therefore potentially contain a range of different immunostimulatory molecules. One obvious advantage of using a complex preparation for vaccine delivery is the ability to trigger several components of the proinflammatory cascade, resulting in broader and more sustained biological activity. In this regard, heat-killed *Brucella abortus* has been recognized as a potent inducer of several components of the immune system including antibody secretion and cytotoxic T lymphocyte responses and has shown promise as a delivery system for human immunodeficiency virus antigens (28). Recently, these characteristics were attributed to the interaction of *B. abortus* with several TLRs and the subsequent involvement of the MyD88-signaling pathway (20). Archaeosomes consisting of different lipid moieties from archaea are another example of a complex adjuvant system triggering different immune responses including cytotoxic responses and antibody production (25, 26). Although many of the specific molecules involved in this signaling cascade remain to be identified, it clearly demonstrates the

TABLE 3. Vaccine-induced protection against an aerosol infection with *M. tuberculosis*

Vaccine[a]	Log_{10} resistance ± SEM[b]	
	Lungs	Spleen
Ag85B-ESAT-6	0.01 ± 0.08	0.14 ± 0.08
Ag85B-ESAT-6 + DDA	0.09 ± 0.15	0.28 ± 0.10
Ag85B-ESAT-6 + BCG lipids	0.48 ± 0.08*	0.47 ± 0.16
Ag85B-ESAT-6 + mycosomes	1.19 ± 0.13*	0.97 ± 0.18*
BCG lipids	0.32 ± 0.07	0.29 ± 0.10
BCG	1.10 ± 0.17*	1.30 ± 0.25*

[a] BALB/c mice ($n = 5$) were immunized three times with the indicated experimental vaccines or injected once subcutaneously with BCG.

[b] Number of bacteria from the lungs and spleen 6 weeks after aerosol challenge expressed as log_{10} resistance calculated by subtracting the log_{10} mean number of bacteria in the organs of vaccinated mice from the log_{10} mean number of bacteria in the organs of naïve mice. Values marked with an asterisk are significantly different ($P < 0.01$) compared to naive controls.

complex interaction between a panel of immunostimulatory molecules from a pathogen and the initiation of a broad and efficient immune response. The identification of receptors through which an innate immune response is triggered is a field in very rapid development (21, 34), and the numbers of identified receptors and, hence, targets for immunomodulation and adjuvant activity are rapidly increasing. An aspect of particular interest in this regard is the role of TLRs in generating and modulating immune responses. Over the last 5 years, this field of research has contributed immensely to our understanding of the mode of action of several of the immunostimulatory molecules that are the backbone of current adjuvant systems. In this regard, both bacterial DNA targeting TLR9 (16) as well as lipid A molecules and synthetically engineered TLR4 agonists have shown promise as vaccine adjuvants (5). The involvement of specific TLRs in relation to the BCG lipid-based adjuvant presented in this paper is the subject of ongoing studies in our laboratory.

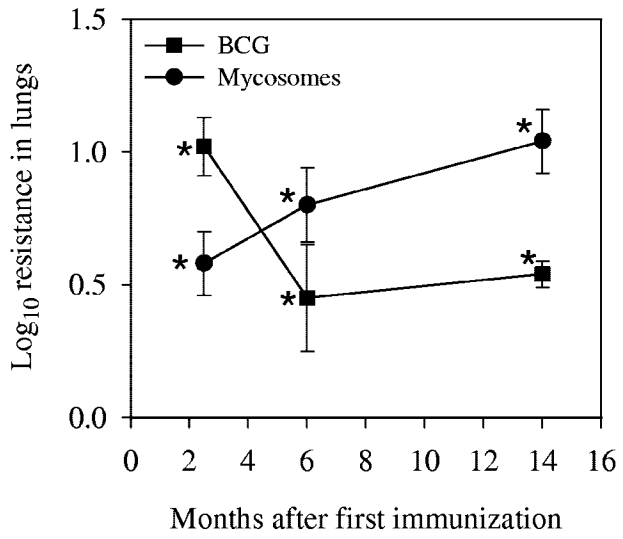

FIG. 6. Immunological memory to TB induced by mycosomes. C57Bl/6 mice ($n = 5$ to 6) immunized with Ag85B-ESAT-6 in mycosomes were challenged with virulent *M. tuberculosis* at 2.5, 6, and 14 months after the first immunization, and the number of CFU ± standard error of the means in lungs was monitored 6 weeks later. Protective effects of the vaccines are expressed as log_{10} resistance calculated by subtracting the log_{10} mean number of bacteria in the lungs of vaccinated mice from the log_{10} mean number in the lungs of unvaccinated control mice. Groups receiving one injection with BCG or nonimmunized mice were included as controls. Bacterial numbers significantly different from those seen in control mice are indicated by an asterisk ($P < 0.01$ as assessed by Dunnetts test).

Mycobacteria have long been known to exert a number of immunomodulatory effects and have been used extensively as a source of adjuvant preparations. The best known adjuvant is Freund's complete adjuvant consisting of a paraffin oil emulsion and heat-killed mycobacteria (15); however, live *M. bovis* BCG has also been used as an immunotherapeutic agent (7). Similarly, purified components of mycobacteria have been shown to have immunostimulatory activity. Wax D (a complex of peptidoglycan, arabinogalactan, and mycolic acids) was found to possess strong adjuvant activity (35), and trehalose 6, 6′-dimycolate (or synthetic analogues thereof) has been included in various adjuvant formulations (19, 24, 30). The use of different preparations of mycobacteria in clinical trials involving cancer patients has demonstrated that they also have a use in this setting (4). Hence, Z-100, a lipid arabinomannan extracted from *M. tuberculosis*, was shown to possess antitumor activity (38) and is now clinically used in Japan. Production of immunostimulatory mycobacterial lipid extracts is feasible, whereas the laborious purification schemes of single molecules currently used for therapeutic applications may be too expensive for a future adjuvant formulation for prophylactic use worldwide. Thus, we have used a simple extraction procedure with a starting material already administered extensively worldwide, the BCG vaccine. In addition to accessibility, another advantage of using BCG would be to avoid immunosuppressive molecules associated with clinical isolates of *M. tuberculosis*. In this regard, a phenolic glycolipid identified from the Beijing strain was recently shown to inhibit the release of proinflammatory cytokines (36). At present, optimized BCG extraction protocols and characterization methods for analysis of stability and batch-to-batch variation are being developed in our laboratory to meet the recommendations in the European Medicines Agency guideline on adjuvants in vaccines (14).

In a recent study, liposomes based on phosphatidylinositol mannosides (PIMS) extracted from BCG were investigated as a potential antigen delivery system. The PIMs were able to activate human dendritic cells, and mice immunized with ovalbumin emulsified in PIM liposomes generated ovalbumin-specific antibody and cytotoxic T-cell responses (37). As described in the present paper, we also tried to use the mycobacterial lipids on their own but found a markedly stronger immune response induced when the mycobacterial lipids were administered in combination with cationic liposomes. Indeed, compared to other liposomes, the cationic surfactant DDA stood out as the most efficient vehicle in terms of both antibody production and IFN-γ levels induced. Although DDA has been used as an adjuvant for many decades (for a review, see reference 18) and has even been administered to humans (41), its specific function as an adjuvant is still not fully understood. In the transfection field DDA has proved a very valuable facilitator of gene uptake and is thought to interact via its positive charge with negatively charged cell membranes (43). It may therefore be a similar activity that allows DDA to enhance the

uptake of antigen and the immunomodulatory mycobacterial lipids by antigen-presenting cells.

In the present study one of the most striking abilities of the mycosomes was the very efficient maintenance of immunological memory that even surpassed the memory induced by the live TB vaccine BCG and resulted in efficient protection against TB as late as 14 months postvaccination. In this regard, the formation of a DDA depot ensuring the slow release of antigen has previously been hypothesized as a mechanism by which DDA may function (19). Experiments involving DDA performed by Katz and coworkers showed an antibody response of a longer duration compared to conventional adjuvants such as alum (22), demonstrating the induction of a persistent response with DDA. However, our studies with DDA alone (1) have never resulted in such striking levels of long-term memory as reported in the present study, and, therefore, in addition to the direct effect of DDA, there is undoubtedly an important effect of the mycobacterial lipids. In this regard, a range of mycobacterial lipids, i.e., phthiocerol dimycocerosates and mycolic acids, have been shown to be very resistant to degradation (17), and they may therefore contribute to the long-term effect seen with DDA-BCG lipids in this study. Along these lines, lipid extracts from archaea have also demonstrated high stability and have been found to be resistant to lipase degradation, pH extremes, and temperature variations (12). Indeed, adjuvant preparations based on these lipids (archaeosomes) were found to provide sustained immune responses as assessed by prolonged antibody production (25).

Together with archaeosomes, the present study represents a move toward a less reductionistic approach to adjuvant development. The sustained release of antigen coupled with the continued stimulation of the immune system by the range of stimulatory compounds present in the mycosomes convincingly demonstrates that strong immune responses and long-lived memory are not exclusively provided by live vaccines like BCG but can also be provided by nonreplicating vaccines such as subunit vaccines in an efficient adjuvant.

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